

# Silver ion chromatography for peak resolution enhancement: Application to the preparative separation of two sesquiterpenes using online heart-cutting LC-LC technique

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1	Silver ion chromatography for peak resolution enhancement: Application to the
2	preparative separation of two sesquiterpenes using online heart-cutting LC-LC
3	technique
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#### 29 Abstract

30 Silver ion chromatography, utilizing columns packed with silver ions bonded to silica gel, has 31 proved to be an invaluable technique for the analysis of some positional isomers. In this work, 32 silver ion chromatography by combination with online heart-cutting LC-LC technique for the 33 preparative separation of two sesquiterpenes positional isomers from a natural product was 34 investigated. On the basis of the evaluation that silver ion content impacts on the separation, the 35 laboratory-made silver ion columns, utilizing silica gel impregnated with 15% silver nitrate as 36 column packing materials, were used for peak resolution improvement of these two isomers and 37 the preparative separation of them in heart-cutting LC-LC. The relationship among the maximal 38 sample load, flow rate and peak resolution in the silver ion column were optimized, and the 39 performance of the silver ion column was compared with conventional C<sub>18</sub> column and silica gel 40 column. Based on the developed chromatographic conditions, online heart-cutting LC-LC 41 chromatographic separation system in combination with a silica gel column and a silver ion 42 column that was applied to preparative separation of these two isomers from a traditional Chinese 43 medicine, Inula racemosa Hook.f., was established. The results showed that the online 44 heart-cutting LC-LC technique by combination of a silica gel column and a silver ion column for 45 the preparative separation of these two positional isomers from this natural plant was superior to 46 the preparative separation performed on a single-column system with C<sub>18</sub> column or silica gel 47 column.

48 Keywords: Positional isomers; Argentation; Peak resolution enhancement; Heart-cutting LC-LC;

49 Preparative separation; Natural product

#### 50 1. Introduction

Silver ion chromatography, utilizing columns packed with silver ions bonded to a silica gel or similar substrate, has proved to be an invaluable technique for the analysis of complex triacylglycerol mixtures, fatty acids, lipids and some positional isomers [1-4]. This application based on the mechanism that the different number, or/and geometrical configuration, or/and position of unsaturated C-C bonds provide the different amount of  $\pi$  donors. Since silver ions act as  $\pi$  acceptors while unsaturated C-C bonds act as  $\pi$  donors, causing molecules that contain fewer  $\pi$ donors or do not contain  $\pi$  donors to be eluted firstly on silver ion chromatography [5]. 58 Although silver ion chromatography by a single-column system is inexpensive and easy to 59 operate for the trace separation and determination of active ingredients, or preparative separation 60 compounds from simple sample on small-scale [6], it still restricts to the large-scale preparative 61 separation of compounds from complex natural products. Due to adsorption of excessive 62 impurities, the action of silver ions becomes worse, causing the decrease of column separation 63 efficiency, even resulting in bleeding of silver ions and affecting the UV detector. In addition, a 64 single-column system sometimes has limited sample capacity and poor relative peak separation [7]. 65 Notably, heart-cutting two-dimensional liquid chromatography (LC-LC) offers the probability to 66 improve these drawbacks based on a tandem combination of two independent liquid phase 67 separation systems [8-11].

68 Inula racemosa Hook.f., a medicinal plant, is widely distributed in Europe, Asia and Africa 69 [12]. Its root as a traditional medicine has been most frequently applied for issues related to peptic 70 disorders, phlegm, detumescence, inflammatory and vermifuge [13]. Alantolactone (AL) and 71 isoalantolactone (IS) (Figure.1) are two sesquiterpenes positional isomers and have abundant 72 content in this medicinal plant. Previous studies suggested that they show many biological 73 activities [14-25], especially in killing of cancer cells. In addition, researchers also have examined 74 the relationship between their structural modifications and biological activities [26-28]. These 75 studies suggested that AL and IS may be good potential lead compounds for future anticancer 76 agent development or act as chemical templates for the design, synthesis, and semisynthesis of 77 new substances. Nevertheless, further studies are required in preclinical and clinical applications 78 and to explain their potential role. For these studies, it certainly will need a large number of 79 materials. In the current ways, acquisition of AL and IS from natural plants is a short-cut. 80 Therefore, a robust method for the preparative separation of AL and IS from this medicinal plant 81 may be necessary. Unfortunately, the structural similarity of AL and IS increases the preparative 82 separation difficulty of them from this natural plant. Although the traditional separation methods 83 used in the trace separation and determination of these two compounds have been performed with 84 capillary electrophoresis (CE) [29], gas chromatography (GC) [30] and RP-HPLC [31, 32], when 85 these methods are used to isolate AL and IS from Inula racemosa Hook.f. on a larger scale, it becomes difficult due to the limitation of the resolution between their peaks. Hence, the 86

establishment of an effective method for increase of the peak resolution and its application to their preparative separation from this plant becomes a key problem. Since AL and IS constitute a pair of positional isomers related to C=C bond, silver ion chromatography provides the probability to improve the separation of them (Figure.1). Therefore, the aim of this study was to improve the separation of these two isomers by silver ion chromatography in preparative scale and obtain them from a crude extract of *Inula racemosa* Hook.f. by preparative separation with silver ion chromatography technique in combination with online heart-cutting LC-LC technique.

94 2. Experimental

#### 95 2.1. Materials and reagents

96 The dried roots of *Inula racemosa* Hook.f. were purchased from Bai Ding (Tibet, China).
97 Alantolactone and isoalantolactone were purchased from National Institute of Food and Drug
98 Control for the quantification and qualitative study (Beijing, China). Silver nitrate was of chemical
99 grade (Zhengzhou Kaidi Chemical Products Co., Ltd, Henan, China). Silica gel (5 ~ 10 µm particles
100 and 10 ~ 40 µm particles) was purchased from Qingdao Yida Silica Reagent Factory (Fujian, China).
101 Ethyl acetate, n-hexane, 95% ethanol and acetonitrile reagents were of analytical grade and
102 purchased from Xi'an Chemical Reagent Factory (Shaanxi, China).

#### 103 **2.2. Sample preparation**

The dried roots of *Inula racemosa* Hook.f. were pulverized and sieved through a screen (100 ~ 200 mesh). 100 g of this powder was extracted with 1000 ml of 95% ethanol solution by ultrasonic treatment for 30 min at 45 °C and 100 kHz/450 W (Bandelin Sonorex, Germany) [33] and repeated three times. The extracts were concentrated on a rotavapor at 45 °C (REC32E, Shanghai Yarong Instrument, China). The residues were dissolved in n-hexane/ethyl acetate (100 mL: 70/30, v/v) solution to get sample solutions for the preparative separation, and the contents of AL and IS in this sample solution were 25.62 mg/mL and 31.32 mg/mL, respectively.

111The stock solution for the analysis of AL and IS was prepared by dissolving AL and IS into112n-hexane/ethyl acetate (70/30, v/v) and mixing at concentration containing AL of 25.86 mg/mL

113 and IS of 30.26 mg/mL.

#### 114 **2.3. Column packing**

115 The laboratory-made silver ion column, utilizing silica gel impregnated with silver nitrate as

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116 column packing materials, was prepared for the preparative separation in heart-cutting LC-LC. 117 The silica gel impregnated with silver nitrate was prepared by the method reported previously with 118 slightly modification [34] (Figure 2). To avoid waste and the risk of blindness, the silver ion content 119 in silica gel impregnated with silver nitrate for the impact on the separation of these two isomers 120 was first observed on silver ion TLC plate [34]. Then the sliver ion columns (Stainless steel tube, 121 Jiangsu Hanbang Co. Ltd., China) were slurry packed with ethyl acetate using a CST 122 chromatographic column packing machine (KeSheng Experimental Equipment Co., Ltd., Suzhou, 123 China) according to the operating instructions, and the performance of the prepared silver ion 124 column for the preparative separation of these two isomers was investigated and compared with 125 C<sub>18</sub> column on preparative HPLC instrument (Two Alltech-627 high-pressure pumps equipped 126 with a Model-500 UV-VIS detector, Alltech, USA).

#### 127 **2.4.** Connections of the heart-cutting LC-LC system

128 The connections of the heart-cutting LC-LC system are illustrated in Figure.3. The column temperatures were controlled at 30 °C using an independent QT-330 chromatographic column 129 130 thermostat (Henan Jiuhegongchuang co., Ltd., China). Two NP7000-C high-pressure pumps equipped with a NU3010-C UV-VIS detector (Jiangsu Hanbang Technology Co., Ltd., Jiangsu, 131 132 China) were involved in the first dimension, and the UV detector was set at 260 nm. The system 133 control and data collection were carried out using an EasyChrom-1000 chromatographic 134 workstation. A silica gel column (22 mm i.d. × 250 mm, 5 µm, Grace, USA) as a preparative 135 column was used for the first-dimension separation, while a 22 mm i.d.  $\times$  20 mm silica gel column 136 as pre-column was equipped between the sample loop and the preparative column. In addition, 137 two Alltech-627 high-pressure pumps equipped with a Model-500 UV-VIS detector (Alltech, USA) 138 were used in the second dimension, and the UV detector was set at 260 nm. The system control and 139 data acquisition were performed by using an AllChrom Plus server workstation. The 140 second-dimension separation was performed on a laboratory-made sliver ion column (22 mm i.d.  $\times$ 141 250 mm).

#### 142 **2.5. Procedure for preparative separation**

*First-dimension unit separation*- N-hexane (A)/ethyl acetate (B) (v/v) were employed as the
mobile phase. 10 mL of the sample solution was injected into a 20 mL sample loop while valve A

was set at position B. Then the elution program was carried out by the EasyChrom-1000
chromatographic workstation: 0–15 min (Valve A at position A; Flow rate, 12 mL/min; 85%
A/15% B; When the peak of the fraction containing AL and IS started to emerge, valve B and
valve A were all switched to position B: the fraction containing AL and IS was cut and transferred
onto the silver ion column via the automatic FC-AS-312 component collector), and 15.1–25 min
(Valve A at position B; flow rate, 15 mL/min; 0% A/100% B) and 25.1–30 min (Valve A at

Second-dimension unit separation– When all of the fraction containing AL and IS from the first-dimension column had been completely transferred onto the second-dimension column, the following elution program was run with a mobile phase composition of (A) n-hexane/(B) ethyl acetate: 0–22 min (Valve B at position A; Flow rate, 10 mL/min; 75% A/25% B; AL and IS were automatically collected by the component collector on the second-dimension unit), 22.1–25 min (Valve B at position B; Flow rate, 15 mL/min; 0% A/100% B) and 27.1–32 min (Valve B at position B; Flow rate, 15 mL min<sup>-1</sup>; 75% A/25% B).

#### 159 2.6. Quantitative HPLC analysis

160 The crude *Inula racemosa* Hook.f. extract and the prepared compounds were analyzed by 161 a commercially available sliver ion column (4.6 mm i.d. × 250 mm, ChromSpher Lipids CP28313, 162 Varian, USA) in the analytical HPLC instrument (Waters 2695 HPLC system equipped with a 163 Waters 2487 UV-VIS detector controlled through an Empower chromatographic workstation, 164 Waters, USA). The concentration range of calibration solutions obtained by diluting the stock 165 solutions at the range of 5172.0-323.25 µg/mL for AL and 6052.0-378.25 µg/mL for IS. 166 Chromatographic separation was performed using an isocratic elution at a flow rate of 1.0 167 mL/min with mobile phase 75/25 (n-hexane/ethyl acetate). The column temperature was controlled 168 at 30 °C, and the effluents were monitored at 260 nm by a UV detector.

#### 169 **3. Results and discussion**

#### 170 **3.1. Silver ion content in the improvement of separation**

For the preparative separation of AL and IS, the goal in the present study was to obtain them with high efficiency. Since low sample load reduces the efficiency in preparative separation of AL

and IS, we were concerned about the increase of the maximal sample load. It is well known that

174 the resolution has great influence on the maximal sample load, as well as run times, equilibration 175 times and solvent consumption. With increase of sample load, the resolution decreases [35]. The 176 decreased resolution limits the increase of the maximal sample load and throughput per unit time. 177 Therefore, the enhancement of the resolution between AL and IS may improve the maximal 178 sample load.

179 The amount of silver ion in the silica gel impregnated with silver nitrate is vital for the 180 improvement of the resolution. Therefore, the investigation on the content of silver nitrate may be 181 necessary. TLC technique in the evaluation of the effect of silver ion content is easy and 182 convenient in this study. In the TLC separation, the developing solvent was composed of 183 n-hexane/ethyl acetate (80/20). The TLC plates were sprayed with an ethanol solution containing 184 10% sulfuric acid and then heated in an IR drier until obvious colour appeared. As shown in Fig.4, 185 AL and IS could not be isolated completely on the TLC plates when no silver nitrate added into 186 silica gel (Figure.4-1 and Figure.4-3), whereas these two isomers on TLC plates by using silica gel  $(10 \sim 40 \,\mu\text{m} \text{ particles})$  impregnated with silver nitrate showed a good separation (Figure.4-2 and 187 188 Figure.4-4 - Figure.4-9). Since the level of silver nitrate impregnation recommended has varied 189 from 0.5-40% [36] and the case where we have found 10% of impregnation is more effective for 190 separations of isomers in column separation [37], 6%, 10% and 15% silver nitrate to improve the 191 separation were investigated in the present study. From the obtained thin-layer chromatograms 192 (Figure.4), with the increase of the silver nitrate content, the amount of sample load increased with 193 complete separation. Since 15% silver nitrate facilitated the isolation more effectively than other 194 ratios with the increase of sample load (Figure.4-3 - Figure.4-9), silica gel (5  $\sim$  10 µm particles) 195 impregnated with 15% silver nitrate as column packing materials was prepared in the following 196 study. Whether more than 15% silver nitrate is more effective in preparative separation of these 197 two isomers needs to be explored.

#### 198

#### 3.2. Sliver ion column for the separation of two sesquiterpenes positional isomers

199 Even though these two positional isomers achieved good separation on silver ion TLC plates 200 with 15% of impregnation, whether the preparative separation in HPLC, utilizing silver ion 201 column packed with this impregnation (15% silver nitrate), could acquire improvement was 202 unknown. Based on the TLC analysis, the investigation was first performed on a single

203 laboratory-made 4.6 mm i.d.  $\times$  250 mm sliver ion column with mobile phase 80/20 204 (n-hexane/ethyl acetate, v/v) at the flow rate of 1 mL/min, where it obtained a good peak 205 resolution, and a satisfactory peak shape and separation times within 20  $\mu$ L of the stock solution 206 (Figure.5a), whereas peak resolution decreased when the sample load increased to 80  $\mu$ L of the 207 stock solution (Figure.5b). Since the mobile phase with n-hexane/ethyl acetate (80/20, v/v) 208 shortened their separation times and reduction of ethyl acetate concentration (Such as reduction of 209 5% ethyl acetate) in mobile phase has a great influence on the retention times, the optimization of 210 the mobile phase composition for the increase of sample load was not carried out. Furthermore, 211 considering that reduction of the flow rate may improve the peak resolution and then increase the 212 sample load [38], the mobile phase 80/20 (n-hexane/ethyl acetate, v/v) at the flow rate of 0.8 213 mL/min and 0.6 mL/min were evaluated in the following experiment, respectively. When the flow 214 rate decreased to 0.8 mL/min, a complete resolution could be obtained within 180 µL of the stock 215 solution (Figure.6), while the retention times of these two isomers slightly delayed but there was 216 no obvious changes in the peak shape. When the sample load continued increasing, bad resolution 217 appeared (Figure.6). In a view of this, the flow rate reduced to 0.6 mL/min, in which the maximal 218 sample load increased to  $480 \ \mu L$  of the stock solution with a complete resolution (Figure.5c and 219 Figure.6). However, the peaks seem to be flat and trailing, and the retention times delayed 220 markedly (Figure.5c). The reason may be the decrease in the elution strength of solvent at the low 221 flow rate. Therefore, the mobile phase was optimized for n-hexane/ethyl acetate (75/25), which 222 reduced peaks trailing and retention times (Figure.5d) and given a complete resolution.

Fig.6 shows the relationship among the resolution, sample load and flow rate. With the increase of sample load, the peak resolution decreased. The increased flow rate also reduced the peak resolution and leaded to the decrease of the peak height. Although the decrease of the flow rate made peak width increase slightly, it obtains a good resolution ( $\geq 1.5$ ) with the increase of sample load. When the sample volume increased to 480 µL of the stock solution, the resolution between the AL and IS nearly equals to 1.5 at the flow rate of 0.6 mL/min, so the sample load didn't increase any more.

In order to validate the recovery and repeatability under the developed conditions using the prepared silver column, 480  $\mu$ L of the stock solution was separated on the 4.6 mm i.d.  $\times$  250 mm column with n-hexane/ethyl acetate (75/25) at the flow rate of 0.6 mL/min. By six replicate
experiments, the recovery of AL was 95.3% with RSD of 1.3% and that of IS was 95.1% with
RSD of 1.6%, while the repeatability was determined with the values of RSD of 1.09% and 1.23%.
The results showed a satisfactory recovery and repeatability.

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#### **3.3.** Conventional separation methods

237  $C_{18}$  and silica gel packing materials are commonly used for preparative separation. Evidently, 238 compared with silica gel, silica gel impregnated with silver nitrate achieved good separation with 239 the increase of sample load (Figure.4). To further evaluate the performance of the laboratory-made 240 silver ion column (4.6 mm i.d.  $\times$  250 mm), a same size C<sub>18</sub> column (4.6 mm i.d.  $\times$  250 mm) for 241 preparative separation of AL and IS was compared with the silver ion column (Chromatographic 242 conditions were presented in supplementary material). Although a satisfactory separation achieved 243 on C<sub>18</sub> column using a higher concentration of water in the mobile phase, it sacrificed a lot of time 244 and still had limited sample treatment capacity because of the limitation of the peak resolution. 245 However, the silver ion column reduced the retention times and increased the maximal sample load with good resolution, compared to C<sub>18</sub> column (Table. 1 and Figure.7). 246

#### 247 **3.4.** Application of heart-cutting LC-LC system in the preparative separation

248 The online heart-cutting LC-LC technique was applied to the preparative separation of these 249 two positional isomers from this natural plant. Although AL and IS could not be separated 250 completely with silica gel materials, the fraction of AL and IS achieved good separation 251 (Figure.4-1 and Figure.8a). Therefore, a silica gel columns was equipped on the first-dimension 252 separation to isolate the fraction of AL and IS from the crude extract, while a laboratory-made 22 253 mm i.d.  $\times$  250 mm sliver ion column was selected for the second-dimension separation to further 254 separate AL and IS. The elution conditions both in the first dimension and the second dimension 255 were developed based on the above analysis. The maximal sample load and the flow rate for the 256 selected silver ion column in LC-LC separation were estimated by applying the linear amplification analysis (About 10.9 mL of the stock solution and flow rate of 14 ml/min) [39], and 257 258 then the diameter of the silica gel column used in first-dimension separation was chosen based on 259 the requirement for making full use of the selected silver ion column in the advantage of the high 260 sample load. For this reason, if 11 mL of the crude extract solution can be treated by silica gel 261 column equipped on the first dimension, the sample load amount loaded on the selected silver ion 262 column transferred from the first dimension may be close to its maximal sample load in LC-LC 263 preparative separation. Therefore, two silica gel columns, with different diameters (10 mm i.d.  $\times$ 264 250 mm, 22 mm i.d.  $\times$  250 mm), were tested respectively. The results showed that the 22 mm i.d. 265  $\times$  250 mm column was superior to another one, in which it could treat 10 mL of the crude extract 266 solution with n-hexane/ethyl acetate (85/15, v/v) at the flow of 12 mL/ min (Figure.8b-d). Hence, 267 a 22 mm i.d.  $\times$  250 mm silica gel column with n-hexane/ethyl acetate (85/15, v/v) as eluent at the 268 flow of 12 mL/ min was used for the first-dimension separation.

269 Although 10 mL of the crude extract solution could be treated in the first-dimension 270 separation (Figure.9a), a bad separation appeared in the second-dimension separation with 271 n-hexane/ethyl acetate (75/25, v/v) at the flow of 14 mL/ min. However, when the flow rate in the 272 second-dimension separation decreased to 10 mL/min, the peaks of these two isomers obtained a 273 baseline separation (Figure.9b). In addition, to maintain the column separation performance per 274 unit time, the columns restored by elution using 100% ethyl acetate at the end of separation each 275 time. Using multicycle and consecutive separation, 2.3 g of AL with a purity of 97.8% and 2.8 g 276 of IS with a purity of 98.8% were obtained from of the crude extract within 8.5 hours. In an 277 LC-LC separation model, the preparative separation achieved high-efficiency compared with 278 conventional methods.

Moreover, the crystals obtained from 90% ethanol were analysed by X-ray diffraction. Full crystallographic details for the compounds reported in this paper were deposited in the Cambridge Crystallographic Data Centre as Supplementary Publication No. CCDC 1438651 for AL (1) [40] and No. CCDC 1438652 for IS (2) [41]. Copies of these data can be obtained upon application to the CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK [*http://www.ccdc.cam.ac.uk*].

284 **4.** Conclusion

Silver ion chromatography was demonstrated to be an effective technique for improvement of the separation of AL and IS in preparative scale. Online heart-cutting LC-LC technique by combination of the silica gel column and silver ion column was successfully applied to preparative separation of AL and IS from a crude extract of *Inula racemosa* Hook.f. This study offers a rapid and effective approach for the preparative separation of these two positional isomers from natural 290 product.

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295 **References** 

- [1] G. Dobson, W.W. Christie, B. Nikolovadamyanova, Silver ion chromatography of lipids and
- 297 fatty acids, J. Chromatogr. B. 671(1995)197-222.
- 298 [2] R. Adlof, Analysis of triacylglycerol and fatty acid isomers by low-temperature silver-ion high
- 299 performance liquid chromatography with acetonitrile in hexane as solvent: Limitations of the
- 300 methodology, J. Chromatogr. A. 1148(2007) 256-259.
- 301 [3] P.P. Daramwar, P.L. Srivastava, B. Priyadarshini, H.V. Thulasiram, Preparative separation of
- 302  $\alpha$  and  $\beta$ -santalenes and (Z)- $\alpha$  and (Z)- $\beta$ -santalols using silver nitrate-impregnated silica gel 303 medium pressure liquid chromatography and analysis of sandalwood oil, Analyst, 137 304 (2012)4564-4570.
- 305 [4] R.O. Adlof, Analysis of triacylglycerol positional isomers by silver ion high performance
  306 liquid chromatography, J. Sep. Sci. 18 (2015)105-107.
- 307 [5] O.K. Guha, J. Janak, Charge-transfer complexes of metals in the chromatographic separation
  308 of organic compounds, J. Chromatogr. A. 68 (1972)325-343.
- [6] R.O. Adlof, Separation of conjugated linoleic acid methyl esters by silver-ion high
  performance liquid chromatography in semi-preparative mode, J. Chromatogr. A. 1033(2004),
  369-71.
- 312 [7] D.R. Stoll, X. Wang, P.W. Carr, Comparison of the practical resolving power of one- and
- two-dimensional high-performance liquid chromatography analysis of metabolomic samples, Anal.
  Chem. 80(2008)268-78.
- 315 [8] X.M. Xie, W.Y. Sun, J.Y. Huang, N. Polachi, L. Tong, et al., Preparative High Performance
- 316 Liquid Chromatography-based Multidimensional Chromatography and Its Application in
- 317 Traditional Chinese Medicine, Chin. J. Anal. Chem. 44(2016)1140-1147.
- 318 [9] M. Pursch, S. Buckenmaier, Loop-based multiple heart-cutting two-dimensional liquid

- 319 chromatography for target analysis in complex matrices, Anal. Chem. 87(2015)5310-7.
- 320 [10] H. Wang, T. Xu, J. Yuan, The use of online heart-cutting high-performance liquid
- chromatography coupled with linear ion trap mass spectrometry in the identification of impurities
  in vidarabine monophosphate, J. Sep. Sci. 40 (2017)1674.
- [11] L.M. de Souza, T.R. Cipriani, C.F. Sant'Ana, M. Iacomini, P.A.J. Gorin, Heart-cutting
  two-dimensional (size exclusion × reversed phase) liquid chromatography-mass spectrometry
  analysis of flavonol glycosides from leaves of Maytenus ilicifolia, J. Chromatogr. A.
- 326 1216(2009)99-105.
- 327 [12] Dictionary CMM. Jiangsu New Medical College: Shanghai People's Press, China, 1977, 244.
- 328 [13] L.R. Song, Dictionary of Modern Chinese Materia Medica, China, 1999, 21, 1-869.
- 329 [14] A. Rasul, M. Khan, M. Ali, J. Li, X.M. Li, Targeting apoptosis pathways in cancer with
- alantolactone and isoalantolactone, Sci. World J. 2013(2013) 248532.
- 331 [15] J. Chun, J.C. Ran, S. Khan, D.S. Lee, Y.C. Kim, et al., Alantolactone suppresses inducible
- 332 nitric oxide synthase and cyclooxygenase-2 expression by down-regulating NF-κB, MAPK and
- 333 AP-1 via the MyD88 signaling pathway in LPS-activated RAW 264.7 cells, Int.
- 334 Immunopharmacol. 14(2012)375–383.
- 335 [16] T.J. Schmidt, R. Brun, G. Willuhn, S.A. Khalid, Antitrypanosomal activity of helenalin and
- some structurally related sesquiterpene lactones, Planta Med. 68(2002), 750–751.
- 337 [17] P.D. Lokhande, K.R. Gawai, K.M. Kodam, B.S. Kuchekar, A.R. Chabukswar, et al.,
- 338 Antibacterial Activity of Isolated Constituents and Extract of Roots of Inula racemosa, Res. J.
- 339 Med. Plant. 1(2007)7-12.
- [18] F. Grimaud, Les Astéracées du Ladakh dans la médecine tibétaine, Phytothérapie. 7(2009)
  255-261.
- [19] J. Qiu, H. Xiang, C. Hu, Q. Wang, J. Dong, *et al.*, Subinhibitory concentrations of farrerol
  reduce α-toxin expression in Staphylococcus aureus, FEMS Microbiol. Lett. 315(201)129-133.
- 344 [20] J. Zhang, Y. Li, D. Duan, J. Yao, K. Gao, et al., Inhibition of thioredoxin reductase by
- alantolactone prompts oxidative stress-mediated apoptosis of HeLa cells, Biochem. Pharmacol.
- 346 102(2016)34-44.
- 347 [21] X.G. Mi, Z.B. Song, P. Wu, Y.W. Zhang, L.G. Sun, et al., Alantolactone induces cell

- apoptosis partially through down-regulation of testes-specific protease 50 expression, Toxicol.
  Lett. 224(2014)349-355.
- 350 [22] Y. Yao, D. Xia, Y. Bian, Y. Sun, F. Zhu, et al., Alantolactone induces G1 phase arrest and
- 351 apoptosis of multiple myeloma cells and overcomes bortezomib resistance, Apoptosis An
- 352 International Journal on Programmed Cell Death. 20(2015)1122-1133.
- 353 [23] H. Cai, X. Meng, Y. Li, C. Yang, Y. Liu, Growth Inhibition Effects of Isoalantolactone on
- 354 K562/A02 Cells: Caspase-dependent Apoptotic Pathways, S Phase Arrest, and Downregulation of
- 355 Bcr/Abl, Phytother. Res. 28(2015)1679-1686.
- 356 [24] J.Y. Seo, J. Park, H.J. Kim, I.A. Lee, J.S. Lim, Isoalantolactone from Inula helenium Caused
- 357 Nrf2-Mediated Induction of Detoxifying Enzymes, J. Med. Food. 12(2009)1038-1045.
- 358 [25] J.Y. Seo, S.S. Lim, J.R. Kim, Nrf2-mediated induction of detoxifying enzymes by
- alantolactone present in Inula helenium, Phytother. Res. 2008(11)1500-5.
- 360 [26] C.L. Cantrell, J.W. Pridgeon, F.R. Fronczek, J.J. Becne, Structure Activity Relationship
- 361 Studies on Derivatives of Eudesmanolides from *Inula helenium* as Toxicants against Aedes 362 aegypti Larvae and Adults, Chem. Biodiversity. 7(2010)1681-1697.
- 363 [27] S.G. Klochkov, S.V. Afanas'Eva, A.B. Ermatova, A.V. Chudinov, Modification of
  364 alantolactones by natural alkaloids, Chem. Nat. Compd. 47(2011)716-725.
- [28] R.X. Guo, L.G. Li, M.L. Zhang, F. Sauriol, Q.W. Shi, et al. Structural modification of
  isoalantolactone and biological activity against the hepatoma cell lines, Heterocycl. Commun.
  20(2014)117-121.
- [29] K. Wang, H. Liu, Y. Zhao, X. Chen, Z. Hul, *et al.*, Separation and determination of
  alantolactone and isoalantolactone in traditional Chinese herbs by capillary electrophoresis,
  Talanta. 52(2000)1001-1005.
- [30] Y. Zhang, G. Xu, R. Jin, L. Xu, Determination of alantolactone and isoalantolactone Inula
  herbs by gas chromatography, China Pharm. Univ. 24(1993)248-250.
- 373 [31] C. Guo, S. Zhang, S. Teng, K. Niu, Simultaneous determination of sesquiterpene lactones
- isoalantolactone and alantolactone isomers in rat plasma by liquid chromatography with tandem
- mass spectrometry: Application to a pharmacokinetic study, J. Sep. Sci. 37(2014)950-956.
- 376 [32] M. Sharma, Separation of isoalantolactone and alantolactone in Inula racemose root by

- 377 RP-HPLC, Pharm. Sin. 2 (2011)6-10.
- 378 [33] A. Trendafilova, C. Chanev, M. Todorova, Ultrasound-assisted extraction of alantolactone
- and isoalantolactone from Inula helenium roots, Pharmacogn. Mag. 6(2010)234-7.
- 380 [34] T.S. Li, J.T. Li, H.Z. Li, Modified and convenient preparation of silica impregnated with
- 381 silver nitrate and its application to the separation of steroids and triterpenes, J. Chromatog. A. 715

382 (1995)372-375

- [35] J.J. Destefano, J.J. Kirkland, Preparative high-performance liquid chromatography, Anal.
  Chem. 47(1975)1193-1175.
- [36] L.J. Morris, Separations of lipids by silver ion chromatography, J. Lipid Res.
  7(1966)717-732.
- 387 [37] A. Cert, W. Moreda, New method of stationary phase preparation for silver ion column
- 388 chromatography: : Application to the isolation of steroidal hydrocarbons in vegetable oils, J.
- 389 Chromatog. A. 823 (1998)291-297.
- 390 [38] J Wang, J Jia, A Aubry, M Arnold, M Jemal. Theory-guided efficient strategy to maximize
- 391 speed and resolution in rapid gradient LC-MS/MS bioanalysis, J. Chromatogr. B. 879
- 392 (2011)1917-1926.
- [39] L.Q. Zhu, Linear amplification effective technology of preparation liquid chromatography,
  Mod. Sci. Instr. 5(2001)72-73.
- 395 [40] Yang Yang CCDC 1438651: Experimental Crystal Structure Determination, 2015, DOI:
  396 10.5517/cc1k9130.
- 397 [41] Yang Yang CCDC 1438652: Experimental Crystal Structure Determination, 2015, DOI:
  398 10.5517/cc1k9141.
- 399



Figure.1 Schematic illustration of sliver ion chromatography for the separation of alantolactone (AL) and



isoalantolactone (IS), and their chemical structures.

Figure.2. Schematic illustration of the preparation of silver ion columns packed with silica gel impregnated with



silver nitrate (SNISG).

Figure 3. Schematic illustration of the instrumental configurations used for online heart-cutting LC-LC preparative

separation.



Figure.4. 0% (1, 3), 6% (4, 7), 10% (5, 8) and 15% (6, 9) silver nitrate as additives: (3-6), 10  $\mu$ L of the stock solution was separated on silver ion TLC plates; (7-9), 20  $\mu$ L of the stock solution was separated on silver ion TLC plates; (1), the crude extract was separated on silica gel TLC plate; (2), the crude extract was separated on silver ion TLC plate (Impregnation with 6% silver nitrate).



Figure.5. (1) AL and (2) IS. (a) 20  $\mu$ L of the stock solution was separated with mobile phase 80/20 (v/v) for n-hexane/ethyl acetate at the flow of 1 mL/min; (b) 80  $\mu$ L of the stock solution was separated with mobile phase 80/20 (v/v) for n-hexane/ethyl acetate at the flow of 1 mL/min; (c) 480  $\mu$ L of the stock solution was separated with mobile phase 80/20 (v/v) for n-hexane/ethyl acetate at the flow of 0.6 mL/min; (d) 480  $\mu$ L of the stock solution was separated with mobile phase 80/20 (v/v) for n-hexane/ethyl acetate at the flow of 0.6 mL/min; (d) 480  $\mu$ L of the stock solution was separated with mobile phase 75/25 (v/v) for n-hexane/ethyl acetate at the flow of 0.6 mL/min; UV 260 nm. Column: Laboratory-made 4.6 mm i.d. × 250 mm sliver ion column.



Figure. 6. The relationship among the resolution, sample load and flow rate. Column: Laboratory-made 4.6 mm i.d.  $\times$  250 mm sliver ion column. Mobile phase: n-hexane/ethyl acetate. Detection: UV 260 nm. Sample: the stock solution.



Figure.7. (1) AL and (2) IS. Chromatogram: Separation of AL and IS with mobile phase 65:35 (v/v) for acetonitrile:water. Flow rate: 0.6 mL/min. Detection: UV 220 nm. Column: Commercially available C18 column. Injection volume: 80  $\mu$ L of the stock solution.



Figure.8. (a) 10  $\mu$ L of the crude extract solution was separated on a 4.6 mm i.d. × 250 mm column with mobile phase 80/20 (v/v) for n-hexane/ethyl acetate at the flow of 1 mL/min. (b) 6 mL of the crude extract solution was separated on a 10 mm i.d. × 250 mm column with mobile phase 80/20 (v/v) for n-hexane/ethyl acetate at the flow of 4 mL/min; (c) 6 mL of the crude extract solution was separated on a 22 mm i.d. × 250 mm column with mobile phase 80/20 (v/v) for n-hexane/ethyl acetate at the flow of 15 mL/min; (d) 10 mL of the crude extract solution was separated on a 22 mm i.d. × 250 mm column with mobile phase 85/15 (v/v) for n-hexane/ethyl acetate at the flow of 12 mL/ min. Detection: UV 260 nm. Column: Commercially available silica gel column.



Figure.9. (1) AL and (2) IS. Chromatogram of online heart-cutting LC-LC separation: (a) the first-dimension separation was carried out on a silica gel column (22 mm i.d.  $\times$  250 mm) with mobile phase 85/15 (v/v) for n-hexane/ethyl acetate at the flow of 12 mL/min, and (b) the second-dimension separation was performed on a laboratory-made sliver ion column (22 mm i.d.  $\times$  250 mm) with mobile phase 75/25 (v/v) for n-hexane/ethyl acetate at the flow of 10 mL/min. Detection: UV 260 nm.